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<b>(21) International Application Number:</b> PCT/US94/12624 <b>(22) International Filing Date:</b> 2 November 1994 (02.11.94)  <b>(30) Priority Data:</b> 08/146,655                      2 November 1993 (02.11.93)                      US  <b>(71) Applicant:</b> GENZYME CORPORATION [US/US]; One Kendall Square, Cambridge, MA 02139 (US).  <b>(72) Inventors:</b> ZIEGLER, Robin, J.; 237 South Street #1, Shrewsbury, MA 01545 (US). LONG, Susan; 8 Pond Street, Boxford, MA 01921 (US).  <b>(74) Agents:</b> GOSZ, William, G. et al.; Genzyme Corporation, One Mountain Road, Framingham, MA 01701-9322 (US).			<b>(81) Designated States:</b> AU, CA, JP, European patent (AT, BE, CH, DE, DK, ES, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE).  <b>Published</b> <i>With international search report.</i>
<b>(54) Title:</b> METHOD OF PRODUCING RECOMBINANT DIMERIC ENZYME			
<b>(57) Abstract</b> <p>This invention relates to a method of producing a recombinant eukaryotic heterodimeric enzyme using a prokaryotic host. The method involves constructing a first DNA vector containing DNA encoding one of the subunits of the dimeric enzyme and then constructing a second DNA vector containing DNA encoding the second subunit of the enzyme. Once the DNA vectors are constructed, they are used to transform a prokaryotic host. The transformed prokaryotic host cell is then cultured under conditions appropriate for the expression of the dimeric enzyme. For example, using the method of the present invention, the heterodimeric isoform of creating kinase CKMB can be produced. This invention further relates to a method of producing a recombinant human dimeric enzyme in an active form using a prokaryotic host, the recombinant enzyme products produced using the method of the present invention, and a transformed prokaryotic host constructed by the method of the present invention.</p>			

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GA	Gabon				

**METHOD OF PRODUCING RECOMBINANT  
DIMERIC ENZYME**

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**Background of the Invention**

The analysis of creatine kinase (CK) isoforms is important for the early diagnosis of acute myocardial infarction (AMI) and for the early determination of coronary artery reperfusion in patients treated with thrombolytic therapy (Alan and Wu, Laboratory Medicine, 23(5):297-302, 1992). The CK isoforms CKMM and CKMB can be used to determine the success of reperfusion therapy, although measurement of MB isoforms provides the earliest and most definitive results. Levels of MM isoforms are also elevated in patients with skeletal muscle disease, and together with the relative MB index, can be useful for determining whether the muscle damage is acute or chronic.

Pure CKMB is needed for research studies of myocardial metabolism and the enzyme's catalytic mechanism and for preparation of standards and quality control materials for clinical analysis. Current methods of obtaining CKMB involve homogenizing heart tissue and precipitating the CKMB with ethanol or ammonium sulfate followed by ion exchange, gel filtration, and/or affinity chromatography (Grace and Roberts, Clin Chem Acta, 123:59-71, 1982; and Herman and Roberts, Anal Biochem, 106:244-252, 1980). These procedures involve multiple column-purification steps, are long and tedious, and may result in poor yields and low specific activities. In addition, the extracts of CKMB can contain large amounts of contaminants, such as albumin which co-fractionates and co-migrates with CKMB on chromatography and pathogens requiring special handling.

A need exists to improve and simplify the production of the different isoforms of CK to satisfy the requirements of researchers and clinicians.

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### Summary of the Invention

This invention relates to a method of producing a recombinant eukaryotic heterodimeric enzyme in an active form using a prokaryotic host. The method involves constructing a first DNA vector containing DNA encoding one of the subunits of the heterodimeric enzyme and, then constructing a second DNA vector containing DNA encoding the second subunit of the enzyme. Once the DNA vectors are constructed they are used to transform a prokaryotic host. The transformed prokaryotic host cell is then cultured under conditions appropriate for the expression of the heterodimeric enzyme. For example, using the method of the present invention, the heterodimeric isoform of creatine kinase, i.e., CKMB, can be produced.

This invention further relates a method of producing a recombinant human dimeric enzyme in an active form using a prokaryotic host.

Furthermore, the present invention relates to the recombinant enzyme products produced using the method of the present invention.

The invention further relates to a transformed prokaryotic host constructed by the method of the present invention.

### Brief Description of the Drawing

Figure 1 a schematic illustration of the method of producing a recombinant dimeric enzyme of the present invention.

### Detailed Description of the Invention

This invention is based upon the discovery that different isoforms of creatine kinase (i.e., CKBB, CKMM and CKMB) can be produced by constructing a DNA vector for the two different subunits of the enzyme and transforming a host cell with the two DNA vectors, the resulting transformed host cell being capable of expressing CKBB, CKMM and CKMB.

#### **DNA vector**

The term "DNA vector" is intended any replication competent vector which has the capability of having a DNA fragment inserted into it and, subsequently, the expression of that DNA insert by an appropriate host

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cell. In addition, the DNA vector must be receptive to the insertion of a DNA fragment containing the DNA where the sequence encodes the subunits of the target eukaryotic dimeric enzyme such as creatine kinase (i.e., CKM and CKB).  
5 Furthermore, the DNA vector must contain a promoter which can be recognized by the host cell. Procedures for the construction of DNA vectors include those described in Maniatis et al., Molecular Cloning, A Laboratory Manual, 2d, Cold Spring Harbor Laboratory Press (1989), herein referred  
10 to as Maniatis et al..

The term "DNA fragment" is intended to encompass any DNA fragment that encodes an enzyme subunit. The DNA fragment once inserted into a DNA vector should be transmittable to a host microorganism by transformation or  
15 conjugation or transfection. Procedures for the construction or extraction of DNA fragments include those described in Maniatis et al. and others known by those skilled in the art.

## 20 Host

The transformed prokaryotic host of the present invention can be created by various methods by those skilled in the art. For example, transfection, transformation or electroporation as explained by Maniatis et al. can be used.  
25 By the term "prokaryotic host" is intended any prokaryote capable of the uptake and expression of foreign DNA, i.e., DNA not originally a part of the prokaryotes's nuclear material. Suitable prokaryotes may include *Corynebacterium*, *Escherichia*, *Streptomyces* or *Bacillus*.

30

## Recombinant Dimeric Enzyme

The recombinant dimeric enzyme of the present invention is intended to encompass any protein consisting of two subunits and possessing enzymatic properties.

35 The invention will be further illustrated by the following non-limiting Exemplification:

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**EXEMPLIFICATION****Materials and Methods:****Cloning CKMB cDNA using PCR**

DNA fragments carrying cDNA encoding CKM and CKB proteins that had been cloned from a human cDNA library have been described (Perryman et al., Biochem. and Biophys. Research Comm., 140: 981-989, 1986; and Villarreal-Levy et al., Biochem. and Biophys. Research Comm., 144:1116-1127, 1987). We used PCR amplification to change the DNA sequence at the N- and C- termini to add restriction enzyme sites that were suitable for cloning the CK cDNA into Genzyme expression vectors. PCR primers were synthesized at Genzyme. The primers had the following 5' - 3' sequences;

SL22 (NdeI site at the ATG start codon of CKB) GCC CAT ATG  
CCC TTC TCC AAC AGC CAC A

SL23 (EcoRI site after the stop codon of CKB) GGA ATT CAT  
TTC TGG GCA GGC ATG AGG

SL24 (NdeI site at the ATG start codon of CKM) GCC CAT ATG  
CCA TTC GGT AAC ACC CAC AAC

SL25 (BamHI site after the stop codon of CKM) GCA GGA TCC  
TAC TTC TGG GCG GGG ATC AT.

The GeneAmp PCR Reagent Kit with AmpliTaq DNA Polymerase from Perkin Elmer Cetus (Norwalk, CT) was used for PCR reactions. The reactions were carried out following standard procedures outlined in the literature enclosed in the kit. Specifically, 5-30 ng DNA, 100 pmol primer DNA, 2.5 U AmpliTaq DNA polymerase, and 200  $\mu$ mol dATP, dCTP, dGTP, dTTP were mixed with supplied buffer, and the reaction mix was overlaid with Ampliwax (Perkin Elmer Cetus). The PCR machine (Coy Laboratory Products, Inc. Grass Lake, MI) was programmed for a cycle of 94°C (melt) for 2 minutes, 55°C (anneal) for 2 minutes, 72°C (extend) for 2 minutes, this cycle was repeated 18 times. A final extension step was run for 10 minutes to allow for complete polymerization of all strands. PCR product (approximately 5-10  $\mu$ g) was digested with NdeI and EcoRI (for CKB) or NdeI and BamHI (for CKM) and purified by electrophoresis through a 0.7% low melting point agarose TAE buffered gel (FMC BioProducts, Inc. Rockland, ME) for cloning into expression vectors. Electrophoresis was performed as described in Molecular Cloning (Sambrook, Fritsch and Maniatis, 1989. Cold Spring Harbor Press, Cold Spring Harbor, NY). Restriction



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endonucleases were purchased from New England BioLabs (Beverly, MA) and digestion reactions set up as suggested by the manufacturer. DNA fragments were purified from gel slices using the Geneclean kit (Bio101, La Jolla, CA).

5

#### Constructing the Genzyme Expression Vector pRZ38

The expression vector was constructed at Genzyme as a derivative of the plasmid pBluescript SK +/- (available from Stratagene, La Jolla, CA). Expression is  
10 driven off the Lac promoter. Our vector, pRZ38, was constructed by adding an restriction enzyme site at the ATG start codon of the b-galactosidase gene through site-directed mutagenesis. Mutagenesis protocols were followed as described using the Muta-Gene In Vitro  
15 Mutagenesis Kit from BioRad (Richmond, CA). This change in the vector allows cloning of foreign genes after the LacZ promoter but maintains similar spacing from the promoter as in the native gene.

#### 20 Cloning CKM and CKB DNA into pRZ38

Vector DNA (5 µg) was digested with NdeI and EcoRI (for CKB) or NdeI and BamHI (for CKM) and gel purified as above. Approximately 100 ng digested vector DNA and 100 ng digested PCR product were ligated in a 20 µl reaction (T4  
25 DNA ligase purchased from NEB, Beverly, MA). Ligation reactions set up as described in Molecular Cloning. After overnight incubation at 15°C ligation mixes were diluted to 60µl with dH2O. 1µl of each was electroporated into electroporation competent E. coli strain MC1061. The  
30 Cell-Porator and Voltage Booster were purchased from Bethesda Research Labs (Bethesda, MD). Protocols for competent cell preparation and electroporation are described in the Instruction manual. Transformants were selected on LB agar plates supplemented with 50 µg/ml ampicillin.  
35 Transformants were analyzed for harboring the correct recombinant plasmid using the alkaline lysis miniprep technique (Molecular Cloning). Miniprep DNA was analyzed by restriction enzyme mapping. 25 ml cultures of pRZ52 (CKB) and pRZ53 (CKM) were grown in LB with 50µg/ml ampicillin and  
40 larger scale DNA preps were purified using QIAGEN plasmid purification columns (QIAGEN Corp., Chatsworth, CA). Dideoxynucleoside chain-termination DNA sequencing reactions

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were carried out according to the standard protocols described in the Sequenase 2.0 kit from USB (Cleveland, OH). [-35S]-dATP (New England Nuclear) was used to radiolabel the sequences for visualization on Kodak XAR film. Reactions  
5 were separated through a 6% polyacrylamide gel, the gel dried, and exposed to film as described in Molecular Cloning. The DNA sequence representing the coding regions of CKB and CKM are in figure G1 and 2.

Once the CKM DNA sequence was determined to be  
10 correct the ampicillin resistance gene in pRZ53 was exchanged for the kanamycin resistance gene from Tn903 (Nomura et al., Gene, 3:39-51, 1987). The ampicillin gene was cut out using the restriction enzymes SspI and BpmI, and the vector ends were blunt ended using T4 polymerase. The  
15 kanamycin resistance gene had been cloned into the polylinker in a pBR322 vector. The gene was cut out of the vector using BamHI and was blunt ended with T4 polymerase. The resulting CKM plasmid is referred to as pRZ69.

## 20 Constructing the CKMB Co-expression Strain

pRZ52 and pRZ69 DNA was mixed together in a concentration of approximately 50 µg/ml and 1 µl of the mixture electroporated into 20 µl MC1061 cells. The cells were plated onto LB plates containing 50 µg/ml ampicillin  
25 and 50 µg/ml kanamycin to select for cells that had been co-transformed with both plasmids. Co-transformants were analyzed by restriction digestion of miniprep DNA (the standard technique is described in Molecular Cloning).



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Analyzing Expression

Expression analysis of the co-transformants was carried out as follows. Clones were grown overnight in 2 ml LB, 0.2% glucose, 50 µg/ml kanamycin and 50 µg/ml ampicillin at 37°C shaking. In the morning 25 ml of LB with 50 µg/ml kanamycin and 50 µg/ml ampicillin was inoculated with 0.6 ml of the overnight culture and grown at 30°C shaking. Cultures were grown to A600 about 0.4 then sampled at one hour intervals for 4 hours. Cultures were left growing overnight and an additional sample taken. Whole cell samples were boiled in SDS-PAGE sample buffer and run through 12% polyacrylamide gels to assess protein production. Gels were obtained from BioRad, and protocols provided with gels were followed.

Several assays were used to determine the level and quality of the expressed recombinant protein. Cultures were grown as previously described. At A600 approximately 1.0 or after overnight incubation cells were harvested and resuspended in a lysis buffer (20 mM Bis-Tris pH 6.9, 0.25% Tween 20, 10 mM b-mercaptoethanol, 10 mM EDTA, 10 mM EGTA, 1mM PMSF). The resuspended cells were lysed by sonication on ice (or for larger scale analysis cells were lysed using the Microfluidizer from Microfluidics Corp., Newton, MA ) and the cell debris removed by centrifugation. Samples were analyzed using the following assay systems after diluting into lysis buffer. The Creatine Kinase Reagent (Sigma 47-UV) is a spectrophotometric assay for kinetic determination of enzyme activity. The Creatine Phosphokinase (CPK) Isoenzymes Kit (Sigma 715-EP) separates the various isoforms (MM, MB, and BB) and stains for activity. The CK-MB assay system for the Abbott IMx analyzer uses a microparticle enzyme immunoassay (MEIA) to determine specific protein mass of CK-MB in a sample. (All protocols are provided with the assay kits.)

Equivalents

Those skilled in the art will recognize, or be able to ascertain, using no more than routine experimentation many equivalents to the specific embodiments of the invention described herein. Such equivalents are intended to be encompassed by the following claims:

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## SEQUENCE LISTING

## (1) GENERAL INFORMATION:

- 5       (i) APPLICANT: Ziegler, Robin J.  
                                Long, Sue
- (ii) TITLE OF INVENTION: Method of Producing  
  Recombinant Dimeric Enzyme
- 10       (iii) NUMBER OF SEQUENCES: 4
- (iv) CORRESPONDENCE ADDRESS:
- 15               (A) ADDRESSEE: Bill Gosz, Esq.,  
  Genzyme Corporation.
- (B) STREET: One Kendall Square
- (C) CITY: Cambridge
- (D) STATE: MA
- (E) COUNTRY: U.S.A.
- 20               (F) ZIP: 02139
- (v) COMPUTER READABLE FORM:
- (A) MEDIUM TYPE: Floppy disk
- 25               (B) COMPUTER: IBM PC compatible
- (C) OPERATING SYSTEM: PC-DOS/MS-DOS
- (D) SOFTWARE: PatentIn Release #1.0,  
  Version #1.25
- (vi) CURRENT APPLICATION DATA:
- 30               (A) APPLICATION NUMBER: US
- (B) FILING DATE:
- (C) CLASSIFICATION:
- (viii) ATTORNEY/AGENT INFORMATION:
- 35               (A) NAME: William G Gosz
- (B) REGISTRATION NUMBER: 27,787
- (C) REFERENCE/DOCKET NUMBER: GEN3-10.0

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## (ix) TELECOMMUNICATION INFORMATION:

(A) TELEPHONE: (617) 252-7583

(B) TELEFAX: (617) 252-7600

(C) TELEX: 201223 GENCAMB

5

## (2) INFORMATION FOR SEQ ID NO:1:

## (i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 1146 base pairs

10 (B) TYPE: nucleic acid

(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

## (ii) MOLECULE TYPE: cDNA

15

## (iii) HYPOTHETICAL: NO

## (iv) ANTI-SENSE: YES

## 20 (v) FRAGMENT TYPE: N-terminal

## (vi) ORIGINAL SOURCE:

(A) ORGANISM: Homo sapiens

## 25 (xi) SEQUENCE DESCRIPTION: SEQ ID NO:1:

ATGCCCTTCT CCAACAGCCA CAACGCACTG AAGCTGCGCT TCCCGGCCGA GGACGAGTTC 60

CCCGACCTGA GCGCCACAA CAACCACATG GCCAAGGTGC TGACCCCGA GCTGTACGCG 120

30

GACGTGCGCG CCAAGAGCAC GCCGAGCGGC TTCACGCTGG ACGACGTCAT CCAGACAGGC 180

GTGGACAACC CGGGCCACCC GTACATCATG ACCGTGGGCT GCGTGGCGGG CGACGAGGAG 240

35 TCCTACGAAG TGTTCAAGGA TCTCTTCGAC CCCATCATCG AGGACCGGCA CCGGCGCTAC 300

AAGCCCAGCG ATGACGACAA GACCGACCTC AACCCCGACA ACCTGCAGGG CGGCGACGAC 360

CTGGACCCCA ACTACGTGCT GAGCTCGCGG GTGGCCACGG GCCGCAGCAT CCGTGGCTTC 420

40

TGCCTCCCCC CGCACTGCAG CCGCGGGGAG CGCCGAGCCA TCGAGAAGCT CGCGGTGGAA 480

- 10 -

GCCCTGTCCA GCCTGGACGG CGACCTGGCG GGCCGATACT ACGCGCTCAA GAGCATGACG 540

GAGGCGGAGC AGCAGCAGCT CATCGACGAC CACTTCCTCT TCGACAAGCC CGTGTCGCCC 600

5 CTGCTGCTGG CCTCGGGCAT GGCCCGCGAC TGGCCCGACG CCGCGCGTAT CTGGCACAAT 660

GACAATAAGA CCTTCCTGGT GTGGGTCAAC GAGGAGGACC ACCTGCGGGT CATCTCCATG 720

CAGAAGGGGG GCAACATGAA GGAGGTGTTC ACCCGCTTCT GCACCGGCCT CACCCAGATT 780

10 GAAACTCTCT TCAAGTCTAA GGACTATGAG TTCATGTGGA ACCCTCACCT GGGCTACATC 840

CTCACCTGCC CATCCAACCT GGGCACCGGG CTGCGGGCAG GTGTCGATAT CAAGCTGCCC 900

15 AACCTGGGCA AGCATGAGAA GTTCTCGGAG GTGCTTAAGC GGCTGCGACT TCAGAAGCGA 960

GGCACAGGCG GTGTGGACAC GGCTGCGGTG GGCGGGGTCT TCGACGTCTC CAACGCTGAC 1020

CGCCTGGGCT TCTCAGAGGT GGAGCTGGTG CAGATGGTGG TGGACGGAGT GAAGCTGCTC 1080

20 ATCGAGATGG AACAGCGGCT GGAGCAGGGC CAGGCCATCG ACGACCTCAT GCCTGCCCAG 1140

AAATGA 1146

25

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## (2) INFORMATION FOR SEQ ID NO:2:

## (i) SEQUENCE CHARACTERISTICS:

- 5 (A) LENGTH: 1146 base pairs  
(B) TYPE: nucleic acid  
(C) STRANDEDNESS: single  
(D) TOPOLOGY: linear

## (ii) MOLECULE TYPE: cDNA

10

## (iii) HYPOTHETICAL: NO

## (iv) ANTI-SENSE: YES

15

## (v) FRAGMENT TYPE: N-terminal

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO:2:

20 ATGCCATTTCG GTAACACCCA CAACAAGTTC AAGCTGAATT ACAAGCCTGA GGAGGAGTAC 60  
CCCGACCTCA GCAAACATAA CAACCACATG GCCAAGGTAC TGACCCTTGA ACTCTACAAG 120  
AAGCTGCGGG ACAAGGAGAT CCCATCTGGC TTTACTGTAG ACGATGTCAT CCAGACAGGA 180  
25 GTGGACAACC CAGGTCACCC CTTCATCATG ACCGTGGGCT GCGTGGCTGG TGATGAGGAG 240  
TCCTACGAAG TTTTCAAGGA ACTCTTTGAC CCCATCATCT CGGATCGCCA CGGGGGCTAC 300  
AAACCCACTG ACAAGCACAA GACTGACCTC AACCATGAAA ACCTCAAGGG TGGAGACGAC 360  
30 CTGGACCCCA ACTACGTGCT CAGCAGCCCG GTCCGCACTG GCCGCAGCAT CAAGGGCTAC 420  
ACGTTGCCCC CACACTGCTC CCGTGGCGAG CGCCGGGCGG TGGAGAAGCT CTCTGTGGAA 480  
35 GCTCTCAACA GCCTGACGGG CGAGTTCAAA GGGAAGTACT ACCCTCTGAA GAGCATGACG 540  
GAGAAGGAGC AGCAGCAGCT CATCGATGAC CACTTCCAGT TCGACAAGCC CGTGTCCCCG 600  
CTGCTGCTGG CCTCAGGCAT GGCCCGCCAC TGGCCCGACG CCCCTGGCAT CTGGCACAAT 660  
40 GACAACAAGA GCTTCCTGGT GTGGGTGAAC GAGGAGGATC ACCTCCGGGT CATCTCCATG 720

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	GAGAAGGGGG GCAACATGAA GGAGGTTTTTC CGCCGCTTCT GCGTAGGGCT GCAGAAGATT	780
	GAGGAGATCT TTAAGAAAGC TGGCCACCCC TTCATGTGGA ACCAGCACCT GGGCTACGTG	840
5	CTCACCTGCC CATCCAACCT GGGCACTGGG CTGCGTGGAG GCGTGCATGT GAAGCTGGCG	900
	CACCTGAGCA AGCACCCCAA GTTCGAGGAG ATCCTCACCC GCCTGCGTCT GCAGAAGAGG	960
	GGTACAGGTG CGGTGGACAC AGCTGCCGTG GGCTCAGTAT TTGACGTGTC CAACGCTGAT	1020
10	CGGCTGGGCT CGTCCGAAGT AGAACAGGTG CAGCTGGTGG TGGATGGTGT GAAGCTCATG	1080
	GTGGAAATGG AGAAGAAGTT GGAGAAAGGC CAGTCCATCG ACGACATGAT CCCC GCCCAG	1140
15	AAGTAG	1146



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## (2) INFORMATION FOR SEQ ID NO:3:

## (i) SEQUENCE CHARACTERISTICS:

5

(A) LENGTH: 381 amino acids

(B) TYPE: amino acid

(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

10

(ii) MOLECULE TYPE: protein

(v) FRAGMENT TYPE: N-terminal

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO:3:

15

Met Pro Phe Ser Asn Ser His Asn Ala Leu Lys Leu Arg Phe Pro Ala  
 1 5 10 15

20

Glu Asp Glu Phe Pro Asp Leu Ser Ala His Asn Asn His Met Ala Lys  
 20 25 30

Val Leu Thr Pro Glu Leu Tyr Ala Asp Val Arg Ala Lys Ser Thr Pro  
 35 40 45

25

Ser Gly Phe Thr Leu Asp Asp Val Ile Gln Thr Gly Val Asp Asn Pro  
 50 55 60

Gly His Pro Tyr Ile Met Thr Val Gly Cys Val Ala Gly Asp Glu Glu  
 65 70 75 80

30

Ser Tyr Glu Val Phe Lys Asp Leu Phe Asp Pro Ile Ile Glu Asp Arg  
 85 90 95

35

His Arg Arg Tyr Lys Pro Ser Asp Asp Asp Lys Thr Asp Leu Asn Pro  
 100 105 110

Asp Asn Leu Gln Gly Gly Asp Asp Leu Asp Pro Asn Tyr Val Leu Ser  
 115 120 125

40

Ser Arg Val Ala Thr Gly Arg Ser Ile Arg Gly Phe Cys Leu Pro Pro  
 130 135 140

- 14 -

	His Cys Ser Arg Gly Glu Arg Arg Ala Ile Glu Lys Leu Ala Val Glu	
	145	150 155 160
5	Ala Leu Ser Ser Leu Asp Gly Asp Leu Ala Gly Arg Tyr Tyr Ala Leu	
		165 170 175
	Lys Ser Met Thr Glu Ala Glu Gln Gln Gln Leu Ile Asp Asp His Phe	
		180 185 190
10	Leu Phe Asp Lys Pro Val Ser Pro Leu Leu Leu Ala Ser Gly Met Ala	
		195 200 205
	Arg Asp Trp Pro Asp Ala Ala Arg Ile Trp His Asn Asp Asn Lys Thr	
		210 215 220
15	Phe Leu Val Trp Val Asn Glu Glu Asp His Leu Arg Val Ile Ser Met	
		225 230 235 240
	Gln Lys Gly Gly Asn Met Lys Glu Val Phe Thr Arg Phe Cys Thr Gly	
20		245 250 255
	Leu Thr Gln Ile Glu Thr Leu Phe Lys Ser Lys Asp Tyr Glu Phe Met	
		260 265 270
25	Trp Asn Pro His Leu Gly Tyr Ile Leu Thr Cys Pro Ser Asn Leu Gly	
		275 280 285
	Thr Gly Leu Arg Ala Gly Val Asp Ile Lys Leu Pro Asn Leu Gly Lys	
		290 295 300
30	His Glu Lys Phe Ser Glu Val Leu Lys Arg Leu Arg Leu Gln Lys Arg	
		305 310 315 320
	Gly Thr Gly Gly Val Asp Thr Ala Ala Val Gly Gly Val Phe Asp Val	
35		325 330 335
	Ser Asn Ala Asp Arg Leu Gly Phe Ser Glu Val Glu Leu Val Gln Met	
		340 345 350
40	Val Val Asp Gly Val Lys Leu Leu Ile Glu Met Glu Gln Arg Leu Glu	
		355 360 365

- 15 -

Gln Gly Gln Ala Ile Asp Asp Leu Met Pro Ala Gln Lys  
370 375 380

5

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## (2) INFORMATION FOR SEQ ID NO:4:

## (i) SEQUENCE CHARACTERISTICS:

- 5 (A) LENGTH: 381 amino acids  
 (B) TYPE: amino acid  
 (C) STRANDEDNESS: single  
 (D) TOPOLOGY: linear

## (ii) MOLECULE TYPE: protein

10

## (v) FRAGMENT TYPE: N-terminal

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO:4:

15 Met Pro Phe Gly Asn Thr His Asn Lys Phe Lys Leu Asn Tyr Lys Pro  
 1 5 10 15

Glu Glu Glu Tyr Pro Asp Leu Ser Lys His Asn Asn His Met Ala Lys  
 20 25 30

20

Val Leu Thr Leu Glu Leu Tyr Lys Lys Leu Arg Asp Lys Glu Ile Pro  
 35 40 45

25 Ser Gly Phe Thr Val Asp Asp Val Ile Gln Thr Gly Val Asp Asn Pro  
 50 55 60

Gly His Pro Phe Ile Met Thr Val Gly Cys Val Ala Gly Asp Glu Glu  
 65 70 75 80

30 Ser Tyr Glu Val Phe Lys Glu Leu Phe Asp Pro Ile Ile Ser Asp Arg  
 85 90 95

His Gly Gly Tyr Lys Pro Thr Asp Lys His Lys Thr Asp Leu Asn His  
 100 105 110

35

Glu Asn Leu Lys Gly Gly Asp Asp Leu Asp Pro Asn Tyr Val Leu Ser  
 115 120 125

40 Ser Pro Val Arg Thr Gly Arg Ser Ile Lys Gly Tyr Thr Leu Pro Pro  
 130 135 140

- 17 -

	His Cys Ser Arg Gly Glu Arg Arg Ala Val Glu Lys Leu Ser Val Glu	
	145	150 155 160
5	Ala Leu Asn Ser Leu Thr Gly Glu Phe Lys Gly Lys Tyr Tyr Pro Leu	
	165	170 175
	Lys Ser Met Thr Glu Lys Glu Gln Gln Gln Leu Ile Asp Asp His Phe	
	180	185 190
10	Gln Phe Asp Lys Pro Val Ser Pro Leu Leu Leu Ala Ser Gly Met Ala	
	195	200 205
	Arg His Trp Pro Asp Ala Pro Gly Ile Trp His Asn Asp Asn Lys Ser	
15	210	215 220
	Phe Leu Val Trp Val Asn Glu Glu Asp His Leu Arg Val Ile Ser Met	
	225	230 235 240
	Glu Lys Gly Gly Asn Met Lys Glu Val Phe Arg Arg Phe Cys Val Gly	
20	245	250 255
	Leu Gln Lys Ile Glu Glu Ile Phe Lys Lys Ala Gly His Pro Phe Met	
	260	265 270
25	Trp Asn Gln His Leu Gly Tyr Val Leu Thr Cys Pro Ser Asn Leu Gly	
	275	280 285
	Thr Gly Leu Arg Gly Gly Val His Val Lys Leu Ala His Leu Ser Lys	
30	290	295 300
	His Pro Lys Phe Glu Glu Ile Leu Thr Arg Leu Arg Leu Gln Lys Arg	
	305	310 315 320
	Gly Thr Gly Ala Val Asp Thr Ala Ala Val Gly Ser Val Phe Asp Val	
35	325	330 335
	Ser Asn Ala Asp Arg Leu Gly Ser Ser Glu Val Glu Gln Val Gln Leu	
	340	345 350
40	Val Val Asp Gly Val Lys Leu Met Val Glu Met Glu Lys Lys Leu Glu	
	355	360 365

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Lys	Gly	Gln	Ser	Ile	Asp	Asp	Met	Ile	Pro	Ala	Gln	Lys
370						375					380	



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CLAIMS

1. A method of producing an eukaryotic heterodimeric enzyme in an active form, comprising:
  - 5 (a) constructing:
    - (i) a first DNA vector containing DNA encoding a first subunit of the enzyme; and
    - (ii) a second DNA vector containing DNA encoding a second subunit of the enzyme;
  - 10 (b) transforming a prokaryotic host with:
    - (i) the first DNA vector; and
    - (ii) the second DNA vector; and
  - (c) culturing the transformed prokaryotic host under conditions appropriate for the expression of the dimeric enzyme.
- 15
2. The method of Claim 1 wherein the eukaryotic heterodimeric enzyme is mammalian.
- 20 3. The method of Claim 1 wherein the eukaryotic heterodimeric enzyme is a kinase.
4. The method of Claim 3 wherein the kinase is a creatine kinase.
- 25
5. The method of Claim 1 wherein:
  - (a) the first subunit is creatine kinase subunit B and second subunit is creatine kinase subunit M; or
  - (b) the first subunit is creatine kinase subunit M and second subunit is creatine kinase subunit B.
- 30
6. The method of Claim 1 wherein the prokaryotic host is bacterial.
- 35 7. The method of Claim 6 wherein the bacterial host is a *Escherichia*.
8. A method of producing a human dimeric enzyme comprising:
  - (a) constructing:
    - 40 (i) a first DNA vector containing DNA encoding a first subunit of the enzyme; and

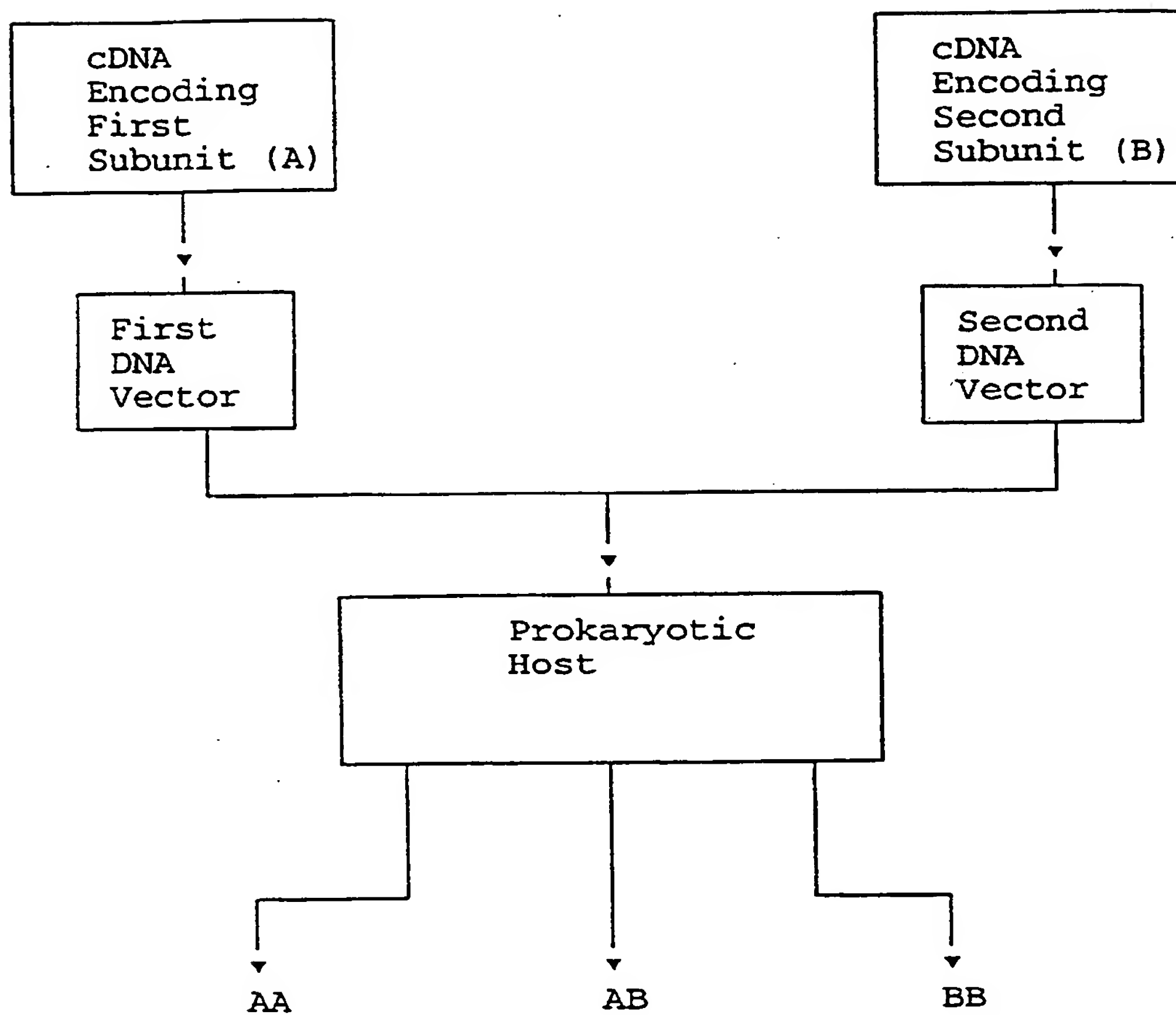
- 20 -

- (ii) second DNA vector containing DNA encoding a second subunit of the enzyme;
  - (b) transforming a prokaryotic host with:
    - (i) the first DNA vector; and
    - (ii) the second DNA vector; and
  - (c) culturing the transformed prokaryotic host under conditions appropriate for the expression of the heterodimeric enzyme.
9. The method of Claim 8 wherein the human dimeric enzyme is a kinase.
10. The method of Claim 9 wherein the kinase is creatine kinase.
11. The method of Claim 8 wherein the prokaryotic host is bacterial.
12. The method of Claim 11 wherein the bacterial host is a *Escherichia*.
13. The method of Claim 8 wherein the first and second subunit are creatine kinase subunit B.
14. The method of Claim 8 wherein the first and second subunit are creatine kinase subunit M.
15. A method of producing a creatine kinase enzyme comprising:
  - (a) constructing:
    - (i) a first DNA vector containing DNA encoding a DNA sequence selected from all or a portion of the DNA sequence of SEQ ID NO.:1; and
    - (ii) a second DNA vector containing DNA encoding a DNA sequence selected from all or a portion of the DNA sequence of SEQ ID NO.:2;
  - (b) transforming a bacterial host with:
    - (i) the first DNA vector; and
    - (ii) the second DNA vector; and
  - (c) culturing the transformed bacterial host under conditions appropriate for the expression of the dimeric enzyme.

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16. A method of producing a creatine kinase enzyme comprising:
- 5 (a) constructing:
- (i) a first DNA vector containing DNA encoding a DNA sequence selected from all or a portion of the DNA sequence of SEQ ID NO.:2; and
- (ii) a second DNA vector containing DNA encoding a DNA sequence selected from all or a portion of the DNA sequence of SEQ ID NO.:1;
- 10 (b) transforming a bacterial host with:
- (i) the first DNA vector; and
- (ii) the second DNA vector; and
- (c) culturing the transformed bacterial host under conditions appropriate for the expression of the dimeric enzyme.
- 15
17. An eukaryotic heterodimeric enzyme produced by the method of Claim 1.
- 20
18. An human dimeric enzyme produced by the method of Claim 8.
19. A creatine kinase enzyme produced by the method of Claim 15.
- 25
20. A creatine kinase enzyme produced by the method of Claim 16.
- 30 21. A transformed prokaryotic host produced by the method of Claim 1.
22. A transformed prokaryotic host produced by the method of Claim 8.
- 35
23. A transformed prokaryotic host produced by the method of Claim 15.
24. A transformed prokaryotic host produced by the method of Claim 16.
- 40

1/1

*Figure 1*

## INTERNATIONAL SEARCH REPORT

International application No.  
PCT/US94/12624

## A. CLASSIFICATION OF SUBJECT MATTER

IPC(6) : C12N 1/21, 9/00, 9/12

US CL : 435/69.1, 183, 194, 252.3

According to International Patent Classification (IPC) or to both national classification and IPC

## B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 435/69.1, 183, 194, 252.3

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

APS, Dialog

search terms: creatine kinase, dimer?, heterodimer?, express?, coexpress?, recombinant

## C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	Journal of Biological Chemistry, Vol. 267, No. 29, issued 15 October 1992, Mayer et al., "CDC43 and RAM2 encode the polypeptide subunits of a yeast type I protein geranylgeranyltransferase", pages 20589-20593, see the entire document.	1-24
Y	Journal of Biological Chemistry, Vol. 266, No. 18, issued 25 June 1991, Chen et al., "Cloning and expression of functional rabbit muscle creatine kinase in <i>Escherichia coli</i> ", pages 12053-12057, see the entire document.	1-24
Y	Clinical Chemistry, Vol. 38, No. 6, issued 1992, Perryman et al., "Human recombinant creatine kinase isoenzyme and subform assay standards", page 981, see the entire document.	1-24

☒ Further documents are listed in the continuation of Box C. ☐ See patent family annex.

* Special categories of cited documents:	* T	later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
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* O* document referring to an oral disclosure, use, exhibition or other means		
* P* document published prior to the international filing date but later than the priority date claimed		

Date of the actual completion of the international search

02 DECEMBER 1994

Date of mailing of the international search report

JAN 30 1995

Name and mailing address of the ISA/US  
Commissioner of Patents and Trademarks  
Box PCT  
Washington, D.C. 20231

Facsimile No. (703) 305-3230

Authorized officer

Eric Grimes

Telephone No. (703) 308-3210

## INTERNATIONAL SEARCH REPORT

International application No.  
PCT/US94/12624

## C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	Biochemical Journal, Vol. 288, issued 1992, Furter et al., "Expression of active octameric chicken cardiac mitochondrial creatine kinase in <i>Escherichia coli</i> ", pages 771-775, see the entire document.	1-24
Y	Clinical Chemistry, Vol. 39, No. 8, issued 1993, Friedman et al., "Recombinant creatine kinase proteins and proposed standards for creatine kinase isoenzyme and subform assays", pages 1598-1601, see the entire document.	1-24



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